

Isolation and Partial Characterization of a High Molecular Weight Cd/Zn-Binding Protein from the Kidney of the Scallop *Placopecten magellanicus*: Preliminary Studies

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Exposure of the scallop *Placopecten magellanicus* to 20 ppb Cd^{2+} in seawater for 7 weeks results in a 7-fold increase in the kidney cytosol content of Cd and 5-fold increase in Zn. Sephadex G-75 column chromatography of the kidney cytosol showed that most of the Cd and Zn were bound to a protein complex with an estimated molecular mass of 45,000 daltons. Further purification of this complex by DEAE A-25 column chromatography disclosed the presence of five peaks with varying degrees of affinity for the ion-exchange resin. One of these peaks (III) was successfully rechromatographed by ion-exchange chromatography and further purified by HPLC using a gel-permeation column. The resultant protein peak which was resistant to disaggregation by 20 mM dithiothreitol gave a preliminary amino acid composition with cysteine, glycine, alanine, and lysine as the major amino acids. The aromatic amino acid phenylalanine was also present. The ultraviolet absorption spectrum gave a 250/280 nm ratio of 2.5:1. Metal analysis of the purified protein showed that it contained Cd, Zn, and Cu in ratios of 1:1:1. Results of these studies indicate that scallop kidney produces a protein complex which appears to share both similarities with mammalian metallothionein with respect to the presence of both Cd and Zn but different with respect to apparent size, amino acid composition, and ultraviolet absorption spectrum.

Introduction

Many species of marine molluscs have been shown to produce high-affinity metal-binding proteins in response to cadmium exposure (1-6). Most of these proteins appear to bind cadmium via SH-mediated multidentate binding sites (2,7,8) in a manner analogous to mammalian metallothionein (MT) but appear to differ from mammalian MT in size (2,5,6,9), electrophoretic mobility (1), total metal-binding capacity (1,2,7), and amino acid composition (1,2,9).

These data suggest that MT or MT-like proteins are widely distributed in nature (10,11), and that there is apparently considerable variability between these molecules from different species, suggesting a number of different but convergent evolutionary pathways for this family of proteins.

A recent report from this laboratory (5) showed that kidneys from bay scallops (*Argopecten irradians*) con-

tained a soluble cadmium-binding protein peak with an estimated molecular weight of 21,000 by Sephadex column chromatography. This value is about twice that reported for mammalian MT by this method (10) but similar to that reported from MT-like cadmium-binding proteins from mussels (2,6,12). The present study was undertaken to further characterize this protein peak in kidneys of the scallop *Placopecten magellanicus* and to determine whether it was a monomer or represented an aggregated form of lower molecular weight protein components. Such data are of clear potential value in both determining the basic chemical characteristics of this protein but also in the development of hypotheses concerning possible pathways for MT evolution.

Materials and Methods

Treatment of Animals

Specimens of the Atlantic sea scallop (*Placopecten magellanicus*) were collected and placed in a flowing seawater system and exposed to 20 ppb Cd added as

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$\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}$ at a salinity of 25‰ at 10°C for 7 weeks. The animals were then placed in clean flowing seawater for 1 to 2 weeks of depuration. Kidneys were excised and frozen on dry ice prior to storage at -70°C and subsequent tissue preparation procedures.

Tissue Homogenization and Protein Isolation Procedures

The kidneys from untreated controls and Cd-treated scallops were pooled and homogenized on ice using a 1:2 (w/v) ratio in a 10 mM Tris-HCl buffer (pH 7.8) containing 10^{-3} M phenylmethyl sulfonyl fluoride (PMSF). Homogenization was conducted by using a Potter-Elvehjem homogenizer and four strokes of a Teflon pestle rotating at 3800 rpm. The kidney calcium phosphate concretion fraction was isolated by low speed centrifugation over 2.5 M sucrose as previously described (3), and the remaining material was centrifuged at 105,000g for 1 hr in a Beckman model L5-65 ultracentrifuge to obtain a clear cytosolic supernatant, which was applied to a 2.0×40.0 cm Sephadex G-75 column equilibrated with a 10 mM Tris-HCl buffer (pH 7.8) at 4°C with an ISCO type 6 UV monitor placed in the effluent line. Fractions (3mL) were collected. The column was calibrated by using blue dextran and protein standards of transferrin (94,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease (13,700). The elution volumes of these proteins were plotted as logs of molec-

ular weight for calibration purposes. All fractions from the scallop kidney runs were monitored for absorbance at 254 nm with the ISCO type 6 UV monitor and at 280 nm with a Gilford model 250 UV/visible spectrophotometer. Cadmium, copper, and zinc were monitored in the kidney supernatant and all chromatographic fractions by using a Perkin-Elmer Model 305 atomic absorption spectrophotometer operated in the flame mode. Cadmium-containing peak tubes from this column were pooled and applied to a DEAE A-25 ion-exchange column equilibrated with 50mM Tris-HCl buffer (pH 8.6). This column was eluted with a 500-mL 0–0.15 M NaCl gradient which yielded five peaks (see below). These peaks were rechromatographed by DEAE A-25 ion-exchange chromatography by use of the same column and gradient system. Only the peak III was successfully rechromatographed by this method. The other peaks (I, II, IV, V) did not reproducibly bind to the DEAE column during the second chromatography and either appeared in the wash fractions or tightly bound to the column such that they could not be readily released. Peak III was desalted and the molecular weight again estimated by gel-permeation high performance liquid chromatography (HPLC) using an LKB model 2150 series instrument with a 7.5×300 nm TSK-G2000 SW column and a wavelength detector set at 254 nm. Atomic absorption analyses for Cd in collected 0.5 mL fractions were measured by atomic absorption spectroscopy on samples from both untreated control and Cd-treated scallops.

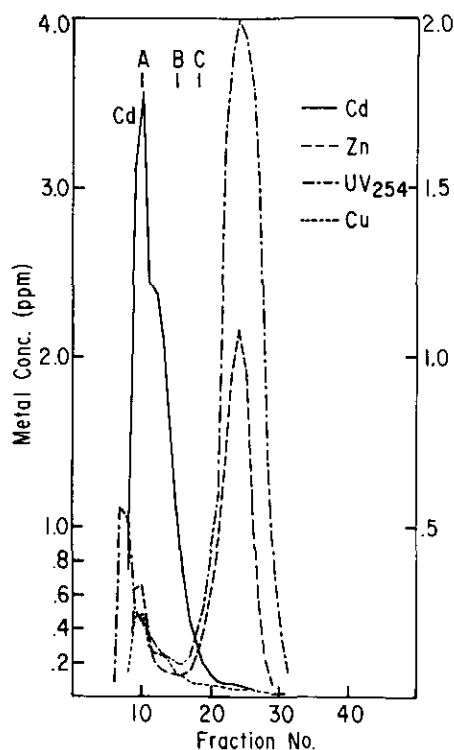


FIGURE 1. Sephadex G-75 chromatography of scallop kidney cytosol showing distribution of Cd, Zn, and Cu in relation to UV absorbance at 254 nm. Molecular weight standards are (A) ovalbumin (45,000), (B) carbonic anhydrase (29,000), and (C) cytochrome c (13,700).

Ultraviolet Absorption Properties

The ultraviolet absorption spectra of the peak III fraction, at pH 8, were measured by using a Gilford model 2600 UV/visible spectrophotometer.

Disaggregation Studies

In order to determine whether the protein could be disaggregated into smaller components, the 45K CdBP peak from control scallops was subjected to incubation with 20 mM dithiothreitol (DTT) at a pH of 8.0 followed by gel-permeation HPLC analysis.

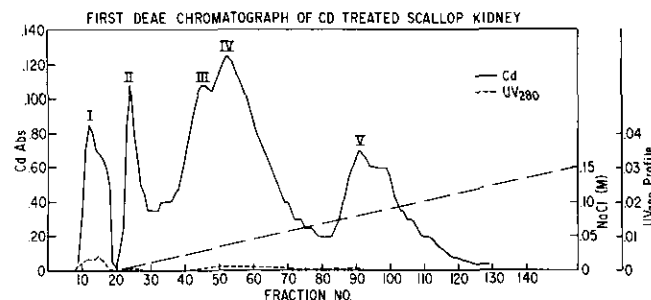


FIGURE 2. First DEAE A-25 chromatograph of 45,000-dalton peak showing five peaks (I–V) with differing affinities for the DEAE resin.

Amino Acid Analyses

Amino acid analyses of protein samples that were homogeneous by HPLC gel-permeation analysis were performed using a Beckman 120 amino acid analyzer. Cysteine determinations were performed following performic acid oxidation according to the method of Hirs (13).

Results

Metal analyses from control and Cd-treated scallop kidney concretion fractions showed an approximately 6-fold increase in the relative concentration of Cd in treated scallops relative to controls, while Cu showed a 50% reduction, and 25% decreases were noted for Zn and Mn. In contrast, total metal analyses of the 105,000g supernatant fraction from treated scallops showed a near 7-fold increase in total Cd and a concomitant 5-fold increase in Zn but 50% decreases in Cu and Mn. These results indicated that metal distribution patterns from the Cd-treatment are differentially affected in these two major intracellular metal-binding compartments (3).

Column Chromatography

G-75 column chromatography of the cytosol fraction from Cd-treated scallops is presented in Figure 1 and shows a marked Cd peak which eluted in the same volume as ovalbumin (A) with a shoulder area extending into lower molecular weight regions. The peak fraction also contained co-incident Zn, Cu, and UV (254 nm) peaks. Initial DEAE A-25-ion-exchange chromatography (Fig. 2) resolved this pooled peak into five Cd peaks (I-V). The first peak (I) did not adhere to the column but appeared in the wash fractions. A second small peak (II) was eluted from the column at the beginning of the gradient while most of the Cd eluted in a major doublet peak (III and

IV) at approximately 0.7 to 0.10 M NaCl. A small Cd peak (V) was also eluted at 0.12 M NaCl. None of the eluted peaks, aside from peak I, possessed appreciable absorbance at 280 nm. Rechromatography of these individual peaks by DEAE-ion-exchange chromatography without desalting showed that Cd associated with peaks I, II, and V could not be eluted from the ion-exchange column by a similar gradient. Replicate studies using peak fractions diluted 2:1 with buffer showed that these peaks could only be eluted with a 0 to 0.5 M NaCl gradient. Rechromatography of peak IV showed that it now eluted in the same position near the beginning of the gradient as peak II on the initial ion exchange chromatography. Only peak III (Fig. 3) eluted near its original position in the first chromatography. This peak was hence carried forward for further analysis.

HPLC gel-permeation chromatography of peak III following the second ion-exchange chromatography, and the 45K CdBP peak from control scallops following similar procedures (Figs. 4 and 5), confirmed that both groups possessed a major Cd-binding peak with a molecular mass of 45,000 daltons, but that the peak III fraction from Cd-treated scallops also contained a 10,000-dalton component which could not be characterized further due to low protein yields. The ratios of Cd:Zn:Cu in this peak were about 1:1:1. This decrease in the measured Cd, Zn, or Cu ratio from that apparent in the G-75 chromatogram appears to result from loss of Cd in the other four peaks observed in the initial DEAE chromatography.

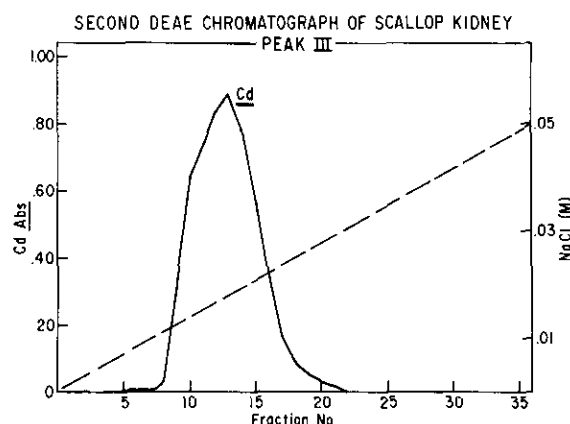


FIGURE 3. Second DEAE A-25 chromatograph of peak III showing single peak early in the NaCl gradient. Recovery of Cd from this chromatography was approximately 100% of the total applied. The Cd-containing peak from G-75 column chromatography of control scallops also eluted in this region of the gradient.

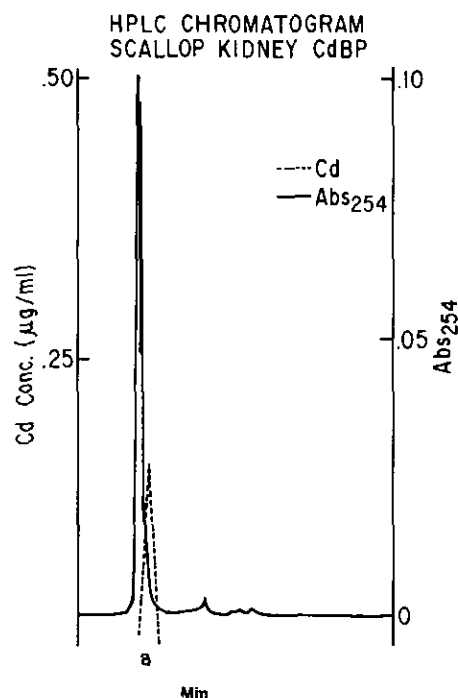


FIGURE 4. HPLC (gel permeation) analysis of the CdBP peak purified by DEAE A-25-ion-exchange chromatography from control scallop kidneys showing a single sharp peak with an apparent molecular mass of 45,000 daltons.

Disaggregation Studies

Attempts to disaggregate the 45K CdBP with 20 mM DTT showed that it was resistant to this procedure. HPLC gel-permeation chromatography of this peak was essentially identical before and after DTT treatment, and no lower molecular weight species were observed.

Ultraviolet Absorption Properties

The ultraviolet absorption spectra of the peak III 45K CdBP is shown in Figure 6 and indicates that this protein has a 250 nm/280 nm ratio of about 2.5:1, which is much slower than that usually observed for mammalian MT (10) but still suggestive of a Cd-S chromophore at 254 nm. Initial circular dichroic studies (data not shown) indicated that this protein did indeed possess a positive band at 259 nm similar to that reported for mammalian (14-16) and avian (16) metallothionein and oyster CdBP (7,8).

Preliminary Amino Acid Analyses

Results of preliminary amino acid analyses conducted on the 45K CdBP from control scallop kidneys showed cysteine (17%) as the major amino acid followed in order by glycine, alanine, and lysine. The aromatic amino acid phenylalanine was also present (Table 1).

Discussion

The results of these studies indicate that kidneys of the scallop *Placochlamys magellanicus* produce a binding

protein (45K CdBP) with an estimated molecular mass of 45,000 daltons that is the major cytosolic depot for Cd, Zn, and Cu under environmental exposure conditions. Zn is primarily associated with a lower molecular weight component with some binding to this protein. Prolonged exposure to a well-tolerated dose of Cd in seawater increases both concentration and cytosolic binding of Cd. In the cytosol fraction, this treatment results in increased binding of both Cd and Zn to the 45K CdBP and increased binding of Cd to a 10,000-dalton protein, which is not present in untreated scallops.

Further purification and partial characterization studies were conducted on the 45K CdBP by combined ion exchange chromatography and HPLC analysis. Results of the initial DEAE-ion-exchange chromatography showed a number of Cd-containing peaks with little absorbance at 280 nm. These chromatographic results are very similar to those reported by Stone et al. (9) for the high molecular weight Cd-binding protein complex from the digestive gland of the scallop *Pecten maximus*. Only peak III of the 45K CdBP protein in this study was successfully rechromatographed by DEAE-ion-exchange chromatography. HPLC gel-permeation analysis of this ion-exchange peak showed a single, sharp Cd-containing peak with a coincident 254 nm absorbance peak in control scallop kidneys. The estimated molecular mass of this peak by this method was similar to that observed by Sephadex G-75 chromatography thus confirming these estimates. In kidneys of Cd-treated scallops, a second Cd-binding peak with a very high coincident 254 nm absorbance and an estimated molecular mass of 10,000 daltons was also observed, suggesting production of a metallothionein.

In order to determine if the 45K CdBP was an aggregate of lower molecular weight species, this protein was incubated with 20 mM dithiothreitol and again subjected to reanalysis by gel-permeation HPLC chromatography. This treatment did not markedly affect the elution volume of the 45K CdBP or result in the disaggregation of this peak, strongly suggesting that this protein was not an aggregate of lower molecular weight forms.

The ultraviolet absorption spectra of the peak III protein showed a metallothionein-like spectra but with a 250 nm/280 nm ratio of only about 2.5:1, which is different

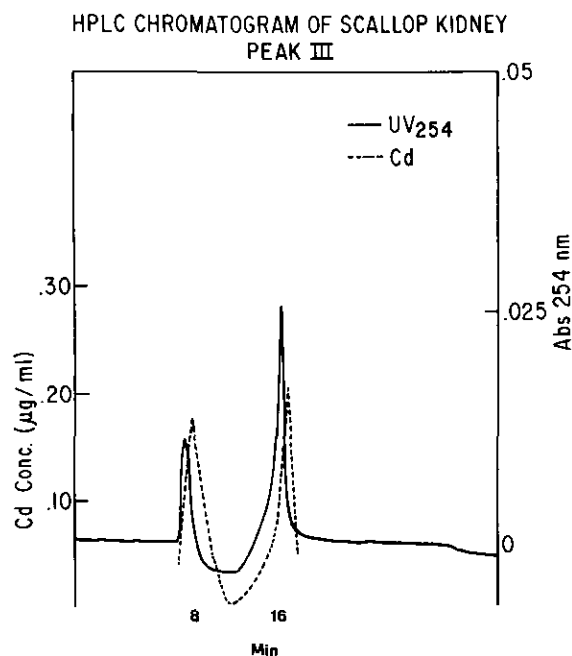


FIGURE 5. HPLC (gel permeation) analysis of peak III from the second DEAE A-25 chromatograph (Fig. 3) showing two peaks at 45,000 and 10,000 daltons.

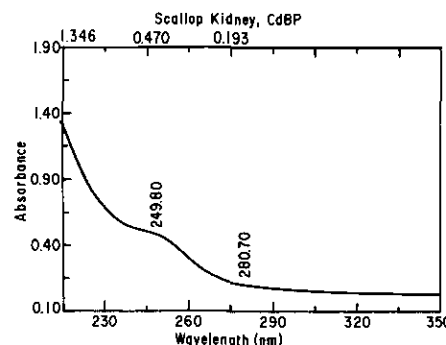


FIGURE 6. Ultraviolet absorption spectrum of the high molecular weight CdBP from scallop kidney.

Table 1. Amino acid composition of scallop kidney CdBP.

Amino acid	Residue, %
Cys	16.8
Asp	7.3
Thr	6.9
Ser	8.0
Glu	7.7
Pro	4.9
Gly	13.0
Ala	10.9
Val	4.5
Met	Trace
Ileu	1.8
Leu	3.1
Tyr	—
Phe	1.8
His	—
Lys	10.7
Arg	2.2

from mammalian MT. Initial circular dichroic studies of this protein, however, confirmed the presence of a positive 259 nm Cd-S chromophore. The major point here is that although apparently much larger than mammalian MT, with a different 250 nm/280 nm ratio, the 45K CdBP possesses apparently similar chemical properties with respect to the 259 nm Cd-S chromophore.

Preliminary amino acid composition studies also suggested that like MT, the 45K CdBP has cysteine as its most abundant amino acid followed by glycine, alanine, and lysine. Phenylalanine was also present. Results of these studies again suggest both similarities and apparent differences between the 45K CdBP with respect to its chemical composition.

In conclusion, the results of these initial studies on the 45K CdBP further confirm the presence of high molecular weight Cd-binding proteins in kidneys of scallops (5,9) and suggest yet another possible evolutionary pathway for MT in higher organisms via gene splitting or gene reduction to yield the 61-residue molecule found in mammals. This hypothesis for the evolution of MT would hence broaden current ideas for MT evolution (11) to include not only gene elongation (11,17) from the smaller thioneins found in microbial organisms but also to include minor amino acid substitutions from proteins of similar size (18) and gene shortening from much larger molecules with similar metal-binding features. The main point here is that we have some evidence to suggest the possibility of convergent evolution for the MT molecule via a number of pathways. It is hoped that in the near future, study of these similar but different metal-binding proteins will yield insight into not only the evolution of MT but also its normal function(s) in biology.

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